

REPORT DOCUMENTATION PAGE			Form Approved OMB NO. 0704-0188	
<small>Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comment regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.</small>				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE		3. REPORT TYPE AND DATES COVERED FINAL
4. TITLE AND SUBTITLE Molecular Genetic Approaches to Biomolecular Materials			5. FUNDING NUMBERS DAAG55-97-1-0354	
6. AUTHOR(S) BAYLEY, Hagan				
7. PERFORMING ORGANIZATION NAMES(S) AND ADDRESS(ES) Texas Engineering Experiment Station 332 Wisenbaker Engineering Research Center College Station, TX 77843-3000			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Research Office P.O. Box 12211 Research Triangle Park,, NC 27709-2211			10. SPONSORING / MONITORING AGENCY REPORT NUMBER ARO 37528.1-LS	
11. SUPPLEMENTARY NOTES The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited.			12 b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) We are using protein engineering to produce biomolecular materials that mimic or extend the properties of materials found in nature. Our current focus is on bacterial S layers and the elastomeric protein abductin. For the most part, these studies are in an exploratory phase. In the case of S layers, we are examining the fundamental properties of the SbsB S layer of <i>Bacillus stearothermophilus</i> by structure-function studies using site-directed mutagenesis and targeted chemical modification. The major finding has been the delineation of a mechanism for the in vitro formation of S layers from their SbsB building blocks. Applications of S layers in biotechnology are also being investigated in parallel with these studies and an extensive cysteine-scanning mutagenesis has been performed to determine sites at which the protein can be chemically modified. In addition, in collaboration with the group of Uwe Sleytr (Vienna), we are using S layers to support bilayers containing genetically engineered pore-forming proteins. These materials will serve as rugged biosensor elements. In the case of abductin, a major advance has been the determination of the sequences of cDNAs encoding the protein.				
14. SUBJECT TERMS			20001124 032	
			15. NUMBER IF PAGES	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OR REPORT UNCLASSIFIED	18. SECURITY CLASSIFICATION OF THIS PAGE UNCLASSIFIED	19. SECURITY CLASSIFICATION OF ABSTRACT UNCLASSIFIED	20. LIMITATION OF ABSTRACT UL	

MOLECULAR GENETIC APPROACHES TO BIOMOLECULAR MATERIALS

HAGAN BAYLEY, PhD

OCTOBER 16, 2000

U. S. ARMY RESEARCH OFFICE

DAAG-55-97-1-0354

TEXAS ENGINEERING EXPERIMENT STATION
332 WISENBAKER ENGINEERING RESEARCH CENTER
COLLEGE STATION, TX 77843-3000

APPROVED FOR PUBLIC RELEASE;
DISTRIBUTION UNLIMITED

THE VIEWS, OPINIONS, AND/OR FINDINGS CONTAINED IN THIS REPORT ARE THOSE OF THE AUTHOR(S) AND SHOULD NOT BE CONSTRUED AS AN OFFICIAL DEPARTMENT OF THE ARMY POSITION, POLICY, OR DECISION, UNLESS SO DESIGNATED BY OTHER DOCUMENTATION.

b. Final Progress Report

- (1) Foreword.- NA
- (2) Table of contents- NA
- (3) List of appendices etc.- NA
- (4) Statement of the problem studied

We are using protein engineering to produce biomolecular materials that mimic or extend the properties of materials found in nature. Our current focus is on bacterial S layers and the molluscan protein abductin. S layers form the outer envelopes of many bacteria. As two-dimensional protein crystals, they are expected to have many interesting applications in biotechnology. Abductin is the major protein constituent of the highly elastic inner hinge ligament of the scallop. The protein, or recombinant derivative of it, will form a basis for the preparations of materials with unusual physical properties.

5. Summary of most important results

- 1) Elucidation of an in vitro assembly pathway for the S layer formed by the SbsB protein of *Bacillus stearothermophilus*. Assembly occurs when the monomeric protein is above a critical concentration, by a nucleation dependent mechanism (Y. Wang).
- 2) Exploration of the domains of SbsB required for S layer assembly by using truncation and deletion mutagenesis. The C terminus is critical for assembly (Y. Wang).
- 3) Development of an improved protocol for high throughput cysteine scanning mutagenesis by PCR and in vivo recombination (S. Howorka).
- 4) Extensive cysteine mutagenesis in preparation for exploring and modifying the properties of SbsB by targeted chemical modification. Surface accessible residues in the monomeric and assembled forms of the bacterial surface layer protein were revealed by modification with a reactive polymer. The identification of residues crucial for assembly was in agreement with and extended the findings from truncation and deletion mutagenesis. (S. Howorka).
- 5) Lipid bilayers supported with S layers. S layers were used to support and thereby strengthen conventional lipid bilayers. Further, S-layer ultrafiltration membranes were developed, providing an even more robust support for bilayers (with B. Schuster in the laboratory of U. Sleytr).
- 6) Sequences of cDNAs encoding the protein component, abductin, of the inner hinge ligament of the mollusk *Argopecten irradians*. Abductin is the major protein constituent of the highly elastic inner hinge ligament. The unusual sequence of the protein was previously unknown and contains a Gly-Gly-Phe-Gly-Gly-Met-Gly-Gly-Gly-Xaa repeat (Q. Cao).
- 7) Visualization of the α -hemolysin pore and an assembly intermediate by atomic force microscopy. This worked confirmed the heptameric structure of the pore under a variety of conditions. It also showed that the prepore assembly intermediate is a

heptamer with its seven-fold axis perpendicular to the membrane surface (ARO project of J. Yang).

- 8) Purification and characterization of recombinant spider silk expressed in *Escherichia coli*. Genes encoding a spider silk repeat sequence were prepared and expressed in *Escherichia coli*. Sufficient material is obtained for studies of fiber processing (with the laboratory of D. Kaplan)

6. List of manuscripts published under ARO sponsorship during this reporting period

Papers:

- 1) Fang, Y., Cheley, S., Bayley, H., and Yang, J. The heptameric prepore of staphylococcal α -hemolysin mutant in lipid bilayers imaged by atomic force microscopy. **Biochemistry** 36, 9518-9522 (1997)
- 2) Cao, Q., Wang, Y., and Bayley, H. Sequence of abductin, the molluscan "rubber" protein. **Current Biology**, 7, R677- R678(1997)
- 3) Cheley, S., Malghani, M.S., Song, L., Gouaux, J.E., Yang, J., and Bayley, H. Spontaneous oligomerization of a staphylococcal α -hemolysin conformationally constrained by removal of residues that form the transmembrane β -barrel. **Protein Engineering**, 10, 1433-1443 (1997).
- 4) Arcidiacono, S., Mello, C., Kaplan, D., Cheley, S., and Bayley, H. Purification and characterization of recombinant spider silk expressed in *Escherichia coli*. **Appl. Microbiol. & Biotechnol.** 49, 31-38 (1998).
- 5) Schuster, B., Pum, D., Braha, O., Bayley, H., and Sleytr, U.B. Self-assembled α -hemolysin pores in an S-layer supported lipid membrane. **Biochim. Biophys. Acta** 1370 280-288 (1998)
- 6) Howorka, S., and Bayley, H. Improved protocol for high throughput cysteine scanning mutagenesis, **Biotechniques** 25, 764-772 (1998).
- 7) Howorka, S., Sára, M., Wang, Y., Kuen, B., Sleytr, U.B., Lubitz, W. and Bayley, H. Surface accessible residues in the monomeric and assembled forms of a bacterial surface layer protein. **J. Biol. Chem.**, in press (2000)
- 8) Schuster, B., Pum, D., Sára, M., Braha, O., Bayley, H. and Sleytr, U.B. S-layer ultrafiltration membranes: a new support for stabilizing functionalized lipid membranes. **Langmuir**, in press (2000)
- 9) Wang, Y., Howorka, S., Kuen, B., Lubitz, W., Sleytr, U.B. and Bayley, H. In vitro assembly pathway of the S layer formed by recombinant and native *Bacillus stearothermophilus* PV72 SbsB protein, in preparation
- 10) Wang, Y., Howorka, S., Kuen, B., Lubitz, W. Sleytr, U.B. and Bayley, H. SbsB protein of *Bacillus stearothermophilus*: effects of truncation on S layer formation, in preparation

Hagan Bayley

Professor and Head

Oxford University, Balliol College
Harvard University
Massachusetts Institute of Technology

BA (1st)	1974	Chemistry
Ph.D.	1979	Chemistry
Postdoc.	1979-1981	Chemistry & Biology

RESEARCH & PROFESSIONAL EXPERIENCE

Professor and Head, Medical Biochemistry and Genetics, Texas A&M University Health Science Center, Professor of Chemistry, 1997-present
Principal Scientist, Worcester Foundation for Biomedical Research, (1994-1996); Senior Scientist, (1988-1994); Associate Professor of Biochemistry & Molecular Biology, University of Massachusetts Medical Center (1991-1996); Associate Professor of Chemistry, Clark University (1996)
Associate Professor, Center for Neurobiology & Behavior, Columbia University (1987-88), and Assistant Investigator, Howard Hughes Medical Institute, Columbia University (1985-88)
University Lecturer in Organic Chemistry, Oxford University (1984-85), and Fellow of Brasenose College, Oxford (1984-85)
Assistant Professor of Biochemistry (1981-84), Columbia University

AWARDS

1970: Open scholarship, Oxford University; 1972: Distinction in Chemical Pharmacology, Oxford University; 1973: Hebertson Prize for Chemistry, Balliol College, Oxford; Gibbs Prize for Chemistry, Oxford University; 1983: Irma T. Hirschl Career Scientist Award.

RESEARCH PROJECTS ONGOING OR COMPLETED DURING THE LAST 3 YEARS

Assembly, structure and function of pore-forming proteins

Principal Investigator; Hagan Bayley, PhD

Present support: DOE (6/15/00-6/14/03), renewal \$480,000

The original goal of this project was to understand the assembly pathway and structure of a pore-forming bacterial toxin, α -hemolysin. Significant progress has been made and at a descriptive level the problem has been to a large extent resolved. Our current efforts have been redirected towards the redesign of α -hemolysin, and the de novo design and *in vitro* evolution of β -barrel membrane proteins. This project uses only molecular genetic approaches, rather than chemical modification, and is therefore distinct from the work proposed here.

Applications of engineered pore-forming proteins

Principal Investigator: Hagan Bayley, PhD

Present support: University of Texas at Austin, Air Force-Multi-site program (6/1/98-11/30/01) \$368,350

Present support: Texas Advanced Technology Program -1999 (1/1/00-12/31/01) \$159,076

Present support: MURI (ONR) - Multisite program project (4/30/99-4/29/04) \$635,073

Pore-forming proteins are being engineered for applications in biotechnology. Our main focus is on the pore-forming bacterial toxin, α -hemolysin. We have used protein engineering to make modified hemolysins with built-in triggers and switches. Pore formation can then be actuated or modulated by biochemical stimuli (e.g. enzyme action), chemical stimuli (e.g. the association and dissociation of metal ions), and physical stimuli (e.g. light). Recent efforts have been directed at using engineered hemolysins as elements in biosensors. New directions include optical and microwave signal detection. Additionally, we have explored the use of engineered hemolysins for the controlled permeabilization of cells, drug delivery and the destruction of malignant cells.

Caged peptides and proteins for signal transduction research

Principal Investigator: Hagan Bayley, PhD

Present support: Welch Foundation (6/1/00-5/31/03) \$135,000

The use of "caged" reagents allows the photogeneration of molecules on or in cells with precise spatial and temporal control. In signal transduction research, effectors and inhibitors can be released at known sites, in defined doses, and at predetermined times. We are using a variety of photoremovable protecting groups to cage peptides and proteins for studies of cell signaling. One tactic we have used has been to derivatize proteins engineered to contain single cysteines at key positions. The activities of many cell signaling proteins are modulated by phosphorylation. Therefore we are also examining peptides and proteins modified on the sulfur atom of thiophosphoryl groups. We are seeking ways other than microinjection to transfer caged proteins to the cell interior based on our knowledge of membrane protein assembly.

Membrane protein engineering by targeted modification

Principal Investigator: Hagan Bayley, PhD

Present support: NIH (3/15/00-2/28/04) \$1,620,000

The properties of α -hemolysin are being re-engineered by using targeted chemical modification. The work differs from the DOE project, which employs direct genetic modification. Targeted modifications include covalent attachment of chelating agents and polymers, as well as non-covalent modification with molecular adapters such as cyclodextrins and cyclic peptides.

SELECTED PUBLICATIONS (total 104)

- Goldman, D.W., Pober, J.S., White, J., and Bayley, H. (1979) Selective labeling of the hydrophobic segments of intrinsic membrane proteins with a lipophilic photogenerated carbene. *Nature* 280:841-843
- Huang, K.-S., Bayley, H., Liao, M.-J., London, E., and Khorana, H.G. (1981) Refolding of an integral membrane protein: Denaturation, renaturation and reconstitution of intact bacteriorhodopsin and two proteolytic fragments. *J. Biol. Chem.* 256: 3802-3809.
- Bayley, H., Huang, K.-S., Radhakrishnan, R., Ross, A.H., Takagaki, Y., and Khorana, H.G. Site of attachment of retinal in bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA* 78:2225-2229 (1981).
- Bayley, H. *Photogenerated Reagents in Biochemistry and Molecular Biology*. Elsevier-North Holland Biomedical Press, 187 pp. (1983).
- Tobkes, N., Wallace, B.A., and Bayley, H. Secondary structure and assembly mechanism of an oligomeric channel protein. *Biochemistry* 24, 1915-1920 (1985).
- Yemul, S.S., Berger, C., Estabrook, A., Suarez, S., Edelson, R., and Bayley, H. Selective killing of T-lymphocytes by phototoxic liposomes. *Proc. Natl. Acad. Sci. USA* 84, 246-250 (1987).
- Bayley, H., Gasparro, F., and Edelson, R. Photoactivatable drugs. *Trends in Pharmacological Sciences* 8, 138-143 (1987).
- Greenberg, S.M., Castellucci, V.F., Bayley, H., and Schwartz, J.H. A molecular mechanism for long-term sensitization in *Aplysia*. *Nature* 329, 62-65 (1987).
- Beushausen, S., Bergold, P., Sturner, S., Roytenberg, V., Schwartz, J.H., and Bayley, H. Two forms of the catalytic subunit of *Aplysia* cAMP-dependent protein kinase arise by alternative RNA splicing. *Neuron* 1, 853-864 (1988).
- Obar, R., Dingus, J., Bayley, H., and Vallee, R. The regulatory subunit, R_{II}, of cAMP-dependent protein kinase binds to a common domain in microtubule associated proteins (MAPs) 2A, 2B, and 2C. *Neuron* 3, 639-645 (1989).
- Beushausen, S., and Bayley, H. A relative of the catalytic subunit of cAMP-dependent protein kinase in *Aplysia* spermatozoa. *Mol. Cell. Biol.* 10, 6775-6780 (1990).
- Cheley, S., and Bayley, H. Kinetics and regulation of two catalytic subunits of cAMP-dependent protein kinase from *Aplysia californica*. *Biochemistry* 30, 10246-10255 (1991).
- Beushausen, S., Lee, E., Walker, B. and Bayley, H. Catalytic subunits of *Aplysia* neuronal cAMP-dependent protein kinase with two different N-termini. *Proc. Natl. Acad. Sci. USA* 89, 1641-1645 (1992).
- Bergold, P., Beushausen, S., Saktor, T., Cheley, S., Bayley, H. And Schwartz, J.H. A regulatory subunit of the cAMP-dependent protein kinase downregulated in *Aplysia* sensory neurons during long-term sensitization. *Neuron* 8, 387-397 (1992).
- Cheley, S., Panchal, R.G., Carr, D.W., Scott, J.R., and Bayley, H. Type II regulatory subunits of cAMP-dependent protein kinase and their binding proteins in the nervous system of *Aplysia*, *J. Biol. Chem.* 269, 2911-2920 (1994).
- Panchal, R.G., Cheley, S., and Bayley, H. Targeting of neuronal substrates by catalytic subunits of *Aplysia* cAMP-dependent protein kinase. *J. Biol. Chem.* 269, 23722-23730 (1994).
- Gouaux, J.E., Braha, O., Hobaugh, M., Song, L., Cheley, S., Shustak, C. and Bayley, H. Subunit stoichiometry of staphylococcal α -hemolysin in crystals and on membranes: a heptameric transmembrane pore. *Proc. Natl. Acad. Sci. USA* 91, 12828-12831 (1994).

PUBLICATIONS FROM 1995 TO DATE (abstracts and reviews excluded)

- Walker, B., Braha, O., Cheley, S. and Bayley, H. An intermediate in the assembly of a protein pore trapped with a genetically-engineered switch. *Chemistry & Biology* 2, 99-105 (1995).
- Chang, C.-Y., Niblack, B., Walker, B. and Bayley, H. A photogenerated pore-forming protein. *Chemistry & Biology* 2, 391-400 (1995).
- Walker, B. and Bayley, H. Restoration of pore-forming activity in staphylococcal α -hemolysin by targeted covalent modification, *Protein Engineering*, 8, 491-495 (1995).

- Walker, B. and Bayley, H. Key residues for membrane binding, oligomerization and pore formation of staphylococcal α -hemolysin identified by cysteine scanning mutagenesis and targeted chemical modification. *J. Biol. Chem.* 270, 23065-23071 (1995)
- Panchal, R. and Bayley, H. Interactions between residues in staphylococcal α -hemolysin revealed by reversion mutagenesis. *J. Biol. Chem.* 270, 23072-23076 (1995).
- Valeva, A., Weiser, A., Walker, B., Kehoe, M., Bayley, H., Bhakdi, S. and Palmer, M. Molecular architecture of a toxin pore: a 15-residue sequence lines the transmembrane channel of staphylococcal α -toxin. *EMBO J.* 15, 1857-1864 (1996).
- Kulkarni, R.S., Zorn, L.J., Anantharam, V., Bayley, H. and Treistman, S.N. The inhibitory effects of ketamine and halothane on recombinant potassium channels from mammalian brain. *Anesthesiology* 84, 900-909 (1996).
- Koltchine, V.V., Anantharam, V., Bayley, H. and Treistman, S.N. Alternative splicing of the NMDAR1 subunit affects modulation of calcium. *Mol. Brain Res.* 39, 99-108 (1996).
- Panchal, R.G., Cusack, E., Cheley, S. and Bayley, H. Tumor protease-activated, pore-forming toxins from a combinatorial library. *Nature Biotechnology* 14, 852-856 (1996).
- Song, L., Hobaugh, M.R., Shustak, C., Cheley, S., Bayley, H. and Gouaux, J.E. Structure of staphylococcal α -hemolysin, a heptameric transmembrane pore. *Science*, 274, 1859-1865. (1996)
- Russo, M., Bayley, H., and Toner, M. Reversible permeabilization of plasma membranes with an engineered switchable pore. *Nature Biotechnology*, 15, 278-282. (1997)
- Pan, P., and Bayley, H. Caged cysteine and thiophosphoryl peptides. *FEBS Letters* 405:81-85 (1997).
- Braha, O., Walker, B., Cheley, S., Kasianowicz, J.J., Song, L., Gouaux, J.E., and Bayley, H. Designed pores as components for biosensors. *Chemistry & Biology*, 7:R677-R678 (1997)
- Fang, Y., Cheley, S., Bayley, H., and Yang, J. The heptameric prepore of a staphylococcal α -hemolysin mutant in lipid bilayers imaged by atomic force microscopy. *Biochemistry*, 36:9518-9522 (1997)
- Valeva, A., Walev, I., Pinkernell, M., Bayley, H., Walker, B., Palmer, M., and Bhakdi, S. (1997) Organization of pore-forming domain of staphylococcal α -toxin differs in susceptible and resistant cell. *Proc. Natl. Acad. Sci. USA*, 94:11607-11611.
- Cao, Q., Wang, Y., and Bayley, H. Sequence of abductin, the molluscan "rubber" protein. *Current Biology*, 7:R677-R678 (1997)
- Cheley, S., Malghani, M.S., Song, L., Hobaugh, M., Gouaux, J.E., Yang, J., and Bayley, H. (1997) Spontaneous oligomerization of a staphylococcal α -hemolysin conformationally constrained by removal of residues that form the transmembrane β -barrel. *Protein Engineering*, 10:1433-1443.
- Arcidiacono, S., Mello, C., Kaplan, D., Cheley, S., and Bayley, H. (1998) Purification and characterization of recombinant spider silk expressed in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 490:31-38.
- Schuster, B., Pum, D., Braha, O., Bayley, H., Sleytr, U. (1998) Self-assembled α -hemolysin pores in an S-layer supported lipid bilayer. *Biochimica et Biophysica Acta* 1370:280-288.
- Chang, C.-Y., Fernandez, T., Panchal, R., and Bayley, H. (1998) A caged catalytic subunit of cAMP-dependent protein kinase. *J. Am Chem. Soc.* 120: 7661-7662
- Howorka, S., and Bayley, H. (1998) Improved protocol for high throughput cysteine scanning mutagenesis, *Biotechniques* 25:764-772.
- Kasianowicz, J.J., Burden, D.L., Han, L.C., Cheley, S., and Bayley, H. (1999) Genetically engineered metal ion binding sites on the outside of a channel's transmembrane β -barrel. *Biophys. J.*, 76:837-845.
- Gu, L.-Q.G., Braha, O., Conlan, S., Cheley, S., Bayley, H. (1999) Stochastic sensing of organic analytes by a pore-forming protein containing a molecular adapter. *Nature*, 398:686-690.
- Cheley, S., Braha, O., Lu, X., Conlan, S. and Bayley, H. (1999) A functional protein pore with a "retro" transmembrane domain. *Protein Science*, 8:1257-1267.
- Eroglu, A., Russo, M.J., Bieganski, R., Fowler, A., Cheley, S., Bayley, H. and Toner, M. (2000) Intracellular trehalose improves the survival of cryopreserved mammalian cells. *Nature Biotechnology* 18:163-167.
- Howorka, S., Movileanu, L., Lu, X., Manon, M., Cheley, S., Braha, O., and Bayley, H. (2000) A protein pore with a single polymer chain tethered within the lumen. *J. Am. Chem. Soc.* 1223:2411-2416.
- Gu, L., Dalla Serra, M., Vincent, J.B., Vigh, G., Cheley, S., Braha, O. and Bayley, H. (2000) reversal of charge selectivity in transmembrane protein pores by using non-covalent molecular adapters. *Proc. Natl. Acad. Sci. USA* 97:3959-3964.

SELECTED REVIEWS FROM 1995 TO DATE

- Bayley, H. Building a door into cells, *Scientific American*, (September issue), pp. 62-67 (1997).
- Bayley, H. Toxin structure: part of a hole? *Current Biology* 7 R763-R767 (1997).
- Bayley, H., Chang, C.-y., Miller, W.T., Niblack, B., and Pan, P. Caged peptides and proteins by targeted chemical modification. *Methods Enzymol.* 291:117-135 (1998).
- Fernandez, T., and Bayley, H. Ferrying proteins to the other side, *Nature Biotechnology*, 16:418-420 (1998).
- Bayley, H. Designed membrane channels and pores, *Curr. Op. Biotechnol.* 10:94-103 (1999).
- Bayley, H. Protein therapy: delivery guaranteed, *Nature Biotechnology* 17:1066-1067 (1999).
- Bayley, H., Braha, O., and Gu, L.-Q. Stochastic sensing with protein pores, *Adv. Mater.* 12:139-142 (2000).

PATENTS (of seven):

- Stochastic sensing mediated by carrier molecules (November 1998, provisional patent filed; November 1999, full patent filed)
- Biosensor compositions and methods of use (February 200, provisional patent filed)